Cell culture isolation and sequence analysis of genetically diverse US porcine epidemic diarrhea virus strains including a novel strain with a large deletion in the spike gene

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ABSTRACT

The highly contagious and deadly porcine epidemic diarrhea virus (PEDV) first appeared in the US in April 2013. Since then the virus has spread rapidly nationwide and to Canada and Mexico causing high mortality among nursing piglets and significant economic losses. Currently there are no efficacious preventive measures or therapeutic tools to control PEDV in the US. The isolation of PEDV in cell culture is the first step toward the development of an attenuated vaccine, to study the biology of PEDV and to develop in vitro PEDV immunoassays, inactivation assays and screen for PEDV antivirals. In this study, nine of 88 US PEDV strains were isolated successfully on Vero cells with supplemental trypsin and subjected to genomic sequence analysis. They differed genetically mainly in the N-terminal S protein region as follows: (1) strains (n = 7) similar to the highly virulent US PEDV strains; (2) one similar to the reportedly US S INDEL PEDV strain; and (3) one novel strain most closely related to highly virulent US PEDV strains, but with a large (197 aa) deletion in the S protein. Representative strains of these three genetic groups were passaged serially and grew to titers of ~5–6 $\log_{10}$ plaque forming units/mL. To our knowledge, this is the first report of the isolation in cell culture of an S INDEL PEDV strain and a PEDV strain with a large (197 aa) deletion in the S protein. We also designed primer sets to detect these genetically diverse US PEDV strains.

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1. Introduction

Porcine epidemic diarrhea virus (PEDV) was first discovered in the US in April–May, 2013 (Cima, 2013). It has now spread to 30 states as of April 23, 2014 and it has subsequently spread throughout North America, including Canada and Mexico (Vlasova et al., 2014). The high

mortality rates (up to 100%) among suckling piglets are associated with significant economic losses. Transmission is through the fecal–oral route. Clinical signs in PEDV infected pigs include severe diarrhea and vomiting leading to dehydration, electrolyte imbalance and death. Before 2013, PEDV was seen only in Asian and European swine herds (Huang et al., 2013; Song and Park, 2012). The PEDV belongs to the genus *Alphacoronavirus* in the subfamily *Coronavirinae* (Marthaler et al., 2013). It has a 28-kb, single stranded, positive sense RNA genome, which is predicted to encode at least seven ORFs in the following order: ORF1a, ORF1b, spike (S), ORF3, envelope (E), membrane (M), and nucleocapsid (N). The spike gene is the most diverse gene (Chen et al., 2013). Currently, two types of PEDV strains are identified in the US: (1) the original highly virulent US PEDV (Stevenson et al., 2013); and (2) the S INDEL PEDV which has insertions and deletions in the N-terminal region of the S protein, with reportedly milder disease in the field (Vlasova et al., 2014; Wang et al., 2014).

The isolation of PEDV in cell culture is the first step toward the development of an attenuated vaccine, as well as a critical achievement to establish *in vitro* virological and immunological assays. For example, a susceptible and robust cell culture system is needed to evaluate disinfection efficiency based on PEDV infectivity *in vitro*. There are previous reports of successful propagation of several non-US PEDV strains using Vero cells and trypsin in the medium (Hofmann and Wyler, 1988; Kusanagi et al., 1992; Song et al., 2003). Shirato et al. (2011) recently reported that trypsin promotes PEDV virion release from infected Vero cells. However, despite numerous reports on detection of PEDV by reverse transcription (RT)-PCR and sequence analysis (Cima, 2013; Huang et al., 2013; Marthaler et al., 2013; Wang et al., 2014), there is only a single report on the successful propagation of the original highly virulent US PEDV strains (Chen et al., 2014). Therefore, we attempted to propagate PEDV from 88 PEDV-positive field samples (63, 14, and 11 intestinal contents, fecal and serum samples, respectively) using Vero cells supplemented with trypsin. We successfully isolated and serially passaged nine US PEDV strains. These PEDV strains showed three distinct genetic profiles in the partial S protein region based on genomic sequence analysis.

2. Materials and methods

2.1. Clinical pig fecal, intestinal, and serum samples

Between May 2013 and February 2014, porcine intestinal contents ($n = 63$), fecal ($n = 14$) and serum ($n = 11$) samples (Table 1) were collected from pigs from farms in several Midwestern States (Ohio, Michigan, Illinois and Iowa) affected by PEDV. The PC168 and PC170, PC173, PC177, PC179, PC180 and PC182, PC21A and PC22A were collected from the same outbreak, respectively. Samples were frozen at $-70\,^{\circ}\mathrm{C}$ until use.

2.2. Gnotobiotic or conventional pig-passaged PEDV samples

The PC21A strain genetically similar to the original highly virulent US PEDV strain, was serially passaged in gnotobiotic pigs four times as described previously by our group (Jung et al., 2014). The small intestinal contents (SIC) from each passage were collected and stored at $-70\,^{\circ}\mathrm{C}$ until use for cell culture. The original pig intestinal contents containing the S INDEL PEDV strain (Iowa106) was collected from a mild diarrhea outbreak case diagnosed at the Veterinary Diagnostic Laboratory, University of Minnesota. Specific pathogen free (SPF) 3-day-old nursing piglets were inoculated orally with $12\,\mathrm{log}_{10}$ genomic equivalents (GE) of the Iowa106 strain, which was filtered using $0.22\,\mu\mathrm{m}$ Millipore filters to remove bacteria. Large intestinal contents (LIC) and SIC were collected from a pig at the acute phase of infection as previously described (Jung et al., 2014) and used for cell culture isolation immediately on the day the samples were collected.

2.3. Cell lines

Several different Vero cell lines, including Vero-81 (ATCC No. CCL-81), Vero 76 (ATCC No. CRL-1587) and Vero-E6 (ATCC No. CRL-1586) were used for PEDV isolation trials. All Vero cells were grown in Dulbecco Modified Eagle Medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with antibiotics (100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of Fungizone®) (Life Technologies), and 5% heat inactivated fetal bovine serum (Hyclone, Logan, UT).

2.4. Preparation of PEDV inoculum for Vero cells

PEDV-positive intestinal or fecal samples were diluted 10-fold in Dulbecco's Phosphate buffered Saline without Mg^{2+} and Ca^{2+} [PBS (−), Sigma, St. Louis, MO], and were vortexed briefly followed by centrifugation at $10,000 \times g$ for 3 min at $4\,^{\circ}\mathrm{C}$. The supernatants were filtered as described above, and then used immediately for inoculation of Vero cells. For serum samples, 10-fold diluted samples were inoculated directly without centrifugation or filtration.

2.5. Virus isolation and propagation

One or two-day-old, semi-confluent Vero cell monolayers were used for virus inoculation. Before inoculation, cells were washed with PBS (−) twice. Then 200 μL, 500 μL and 1 mL of inoculum per well for a 24-well plate, 6-well plate, and for a T_{25} flask, respectively, was added. After incubating at $37\,^{\circ}\mathrm{C}$ for 30–60 min, PEDV growth medium [DMEM supplemented with antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin, Life Technologies), 0.3% tryptose phosphate broth (TPB, Sigma), and 10 μg/mL of trypsin (Life Technologies)] was added (1 mL, 2 mL, and 5 mL per well for a 24-well plate, 6-well plate, and for a T_{25} flask, respectively) without removing the inoculum. This condition was designated as "Inoc (+)" conditions. We also tried another condition which was designated as "Inoc (−)" condition: the inoculum was removed after 30–60 min-incubation, and the cells were washed once with PBS (−), and then the PEDV growth medium was added.

For the first inoculation, Vero cells cultured in 24- or 6-well plates were used. When cytopathogenic effects (CPE)

Table 1
Summary of PEDV strains isolated in Vero cells.

Sample ID	Sample collection date	State-farm	Pig age (days)	Specimen	Virus isolation condition	Passage no. in Vero cells	PEDV RNA titer (log$_{10}$ GE/mL)	PEDV titer Mean ± SD (log$_{10}$ PFU/mL)	Type of N-terminal S protein genetic character
PC22A[a]	2013 June	OH-A	~3	intestinal content or mixture of SIC&LIC	Inoc (+)[i]	30	12.65	5.60 ± 0.13	Similar to US major highly virulent strain
PC168[a]	2013 November	MI-A	1	SIC[g]	Inoc (+)[i]	2	12.13	4.76 ± 0.39	Similar to US major highly virulent strain
PC170[a]	2013 November	MI-A	1	SIC[g]	Inoc (+)[i]	2	12.11	4.58 ± 0.03	Similar to US major highly virulent strain
PC173[a]	2013 December	OH-B	3	SIC[g]	Inoc (+)[i]	2	12.23	3.34 ± 0.37	Similar to US major higlyh virulent strain
PC177[a]	2013 December	OH-B	2	SIC[g]	Inoc (−)[j]	8	12.93	5.12 ± 0.14	197 aa deletion in S protein
PC179[a]	2013 December	OH-B	3	SIC[g]	Inoc (+)[i],(−)[j]	1	NT	NT	NT
PC180[a]	2013 December	OH-B	3	LIC[h]	Inoc (+)[i], (−)[j]	2	12.70	3.64 ± 0.62	Similar to US major highly virulent strain
PC182[a]	2013 December	OH-B	3	LIC[h]	Inoc (+)[i]	2	12.29	3.62 ± 0.45	Similar to US major highly virulent strain
PC21A (PE21)[b]	2013 June[d]	OH-A	11 (1 PID[f])	SIC[g]	Inoc (+)[i]	1	NT	NT	NT
PC21A (PE103)[b]	2013 June[d]	OH-A	38 (3 PID[f])	SIC[g]	Inoc (+)[i]	4	12.52	3.61 ± 0.23	Similar to US major highly virulent strain
Iowa 106 (PV39)[c]	2013 December[e]	IA	6 (3 PID[f])	SIC[g]	Inoc (+)[i]	10	13.21	5.58 ± 0.14	Similar to US mild S INDEL strain

[a] Samples from swine farm.
[b] The 1st passage (PE21) and the 4th passage (PE103) of PC21A strain in germ-free piglets.
[c] The 1st passage of Iowa 106 in one conventional piglet.
[d] Sample collection date for the original field sample, PC21A.
[e] Sample collection date for the original field sample, Iowa106.
[f] Post-inoculation-day.
[g] Small intestinal content.
[h] Large intestinal content.
[i] Curtured without removing the inoculum.
[j] Cultured after inoculum was removed after 30–60 min-incubation, and then the cells were washed once with PBS(−).

of Vero cells appeared in >90% of cells (~7 days after inoculation), the plate was frozen at −70 °C and thawed once. The cells and supernatants were mixed by pipetting, aliquoted and stored at −70 °C. These samples were used as seed stocks for the next passage. For serial passaging, the culture scale was gradually increased, until finally T_{75} flasks were used for propagation and serial passage of PEDV strains.

2.6. Immunofluorescence assay (IFA) for the detection of PEDV antigens in Vero cells

PEDV-infected Vero cells in 24-well plates were fixed with acetone/methanol (20:80, v/v) at −20 °C for 20 min, and then the fixed cells were washed by PBS, and blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h. Mouse anti-PEDV N protein monoclonal antibody #6-29 (a gift from Dr. Steven Lawson, South Dakota State University) and Fluorescence isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Serotec, Oxford, UK) were used as first and second antibodies, respectively. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and then the plates were examined using a fluorescence microscope (Olympus IX-70).

2.7. Electron microscopy (EM) for the detection of PEDV particles

For imaging virions in infected Vero cells, Vero cells culture media was removed and replaced with fixative containing 3% glutaraldehyde, 1% paraformaldehyde in 0.1 M potassium phosphate buffer pH 7.2 (PB). Cells were fixed for 4 hours at room temperature, then washed three times with excess PB, and post-fixed with 1% osmium tetroxide, 1% uranyl acetate in PB for 1 h. After three washes with distilled water, cells were collected, embedded in 0.6% low melting agarose (Fisher Scientific, Pittsburgh, PA #9012-36-6), dehydrated through a graded ethanol-propylene oxide series, and embedded in EM Bed812 resin (Electron Microscopy Sciences, Hartfield, PA). Ultrathin-sections were prepared using a Leica EM UC6 ultra-microtome. After staining with 3% aqueous uranyl acetate for 20 min, followed by Reynolds' lead citrate for 10 min, sections were imaged using a Hitachi H-7500 transmission electron microscope and images recorded with the Optronics QuantiFire S99835 (SIA) digital camera. For imaging virion particles in Vero cell culture media, PEDV-infected Vero cell culture media were clarified by centrifugation at $2000 \times g$ for 30 min, followed by filtration of the supernatants through 0.45 μm filters. These samples were centrifuged at $106,750 \times g$ for 2 h at 4 °C using an ultracentrifuge (Beckman Coulter, Miami, FL). Virus pellets were resuspended in 50 μL of PEDV growth media. 20 μL of sample was applied to a 300-mesh formvar and carbon coated copper grid for 5 min. After blotting, the grid was washed with water and subsequently 30 μL of 3% phosphotungsic acid (PTA, pH7.0) in 0.4% sucrose was added, for 1 min. After blotting and drying, grids were examined using an electron microscope (Hitachi H7500).

2.8. Plaque assay for the titration of PEDV infectivity

We developed a plaque assay for PEDV using procedures similar to those described by Gonzalez-Hernandez et al. (2013). The growth media of confluent cell monolayers in 6-well plates was replaced with maintenance media. Following 1 h incubation at 37 °C, the cells were inoculated in duplicate wells with 10-fold serially diluted virus [0.5 mL/well] and incubated for 1 h at 37 °C. Then, virus inocula were removed and cell monolayers were washed once by PBS (−). Immediately, the cell monolayers were covered by 2 mL/well of an equal volume of 3% SeaPlaque agarose (Lonza, Rockland, ME) and 2× MEM (Life Technologies) containing 1% anti-anti, HEPES, MEM non-essential amino acids (Invitrogen) and 20 μg/mL Trypsin. At 3 PID, 0.01% neutral red (Sigma) in PBS (−) was added, 2 mL/well, for staining. After incubation for a maximum of 3 h, neutral red solution was removed and plaques were visualized and counted within few hours. Infectious viral titers were described as plaque forming units (PFU)/mL.

2.9. RNA extraction

The RNA of intestinal/fecal suspensions and cell culture samples were extracted using the 5× MagMAX-96 Viral 1 Kit (Ambion, Austin, TX) and the RNA extraction robot MagMax^{TM} Express (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

2.10. RT-PCR and TaqMan real-time RT-PCR (RT-qPCR) for the detection of PEDV N protein gene

The presence of PEDV in the samples used for Vero cell culture inoculation was first confirmed by using RT-PCR or RT-qPCR targeting the PEDV N gene, as described previously (Jung et al., 2014). Briefly, forward primer PEDN219 (5′-GCATTTCTACTACCTCGGAACA) and reverse primer PEDN557 (5′-CTCCACGACCCTGGTTATTT) were used in RT-PCR. Forward primer PEDNFnew (5′-CGCAAA-GACTGAACCCACTAAC), reverse primer PEDNR (5′-TTGCCTCTGTTGTTACTTGGAGAT) and probe PED probe (FAM-TGYYACCAYYACCACGACTCCTGC-BHQ3) were for RT-qPCR.

2.11. RT-PCR screening for other enteric viruses

The samples that showed obvious CPE (a characteristic fused-syncytium cell formation) and increased PEDV viral RNA titers (at least 10-fold increase) were serially passaged at least twice to T_{75} flasks and were also confirmed to be negative for rotavirus A, B and C, TGEV, PRCV and caliciviruses by RT-PCR as previously described (Amimo et al., 2013a,b; Kim et al., 2000; Wang et al., 2007) and porcine deltacoronavirus by RT-qPCR targeting the M gene (Marthaler et al., 2014).

2.12. Sequence analysis

Partial PEDV genome cDNA covering 3′-end of the ORF1b and 5′-end of S gene was amplified using primer set

PEDV-20320-F (5′-AACACGTCATCGTCAGAGGC-3′) and PEDV-21816R (5′-CGGTTGGAGGTAAAACAGC-3′), which was designed based on the US PEDV strain Colorado/2013 (Marthaler et al., 2013). PCR products were analyzed by 1% agarose gel electrophoresis. The PCR products generated by RT-PCR were purified by the QIAquick Gel extraction kit (Qiagen, Hilden, Germany), and then directly sequenced by using PEDV-20320-F, PEDV-21004-F (5′-GCATTTGCCAGTTTCCTAG-3′), PEDV-20892-R (5′-CTCTAA-TATGGCTAACAAAG-3′), PEDV-21339-R (5′-CAATGCAAT-TAGCTGTGTAC-3′), and PEDV-21816-R primers. Sequencing analysis was performed using the Big Dye Terminator (version 1.1) Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automated sequencer, the 3100 xl Genetic Analyzer (Applied Biosystems). Nucleotide sequences were assembled with the program Sequencher™, version 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). Nucleotide and amino acid sequences were analyzed with GENETYX® Mac software, version 16.0.4 (Genetyx Corporation, Tokyo, Japan).

Genomic sequences of tissue cultured (TC) PEDV strains [PC177, Iowa106 (PV39), PC22A, PC21A (PE103), PC168, PC170, PC173, PC180, and PC182] were sequenced utilizing Next Generation Sequence Technology at the University of Minnesota Genomic Center, and genomic assembly was conducted as described previously (Marthaler et al., 2013).

Amino acid sequences corresponding to the partial S protein region and nucleotide sequences of genomic sequences were aligned with Clustal W method. Phylogenetic trees were constructed using the Maximum Likelihood method [Jones–Thornton–Taylor (JTT) and General Time Reversible nucleotide substitution models for amino acids and nucleotides, respectively] and supported with a bootstrap test of 1000 replicates in MEGA 6.06 software (Tamura et al., 2013).

3. Nucleotide sequence accession numbers

Genomic sequences of the 9 isolated PEDV strains were submitted to GenBank under accession no. KM392224–KM392232.

4. Results

4.1. Nine US PEDV strains were successfully propagated in Vero cells

For PEDV propagation, we first tested different Vero cell lines. We selected Vero-81 for subsequent experiments because Vero-76 and especially Vero-E6 cells were more sensitive to trypsin in the medium, resulting in cell detachment (Vero-E6 cells) and rounding (Vero-76 cells). However, viral RNA titers of PC22A infected Vero-E6 and Vero-76 cell cultures were similar to those of PC22A infected Vero-81 after initial isolation (data not shown).

We observed typical cytopathic effect (CPE), such as syncytium Vero cells after inoculation with virulent case samples [PC22A, PC168, PC170, PC173, PC179, PC180, PC182, PC21A (PE21 and PE103)], S INDEL sample [Iowa106 (PV39)] and PC177 (197 aa S deletion) (Fig. 1). All of these samples were intestinal contents [small intestinal contents (SIC), large intestinal contents (LIC) or mixtures] (Table 1). We tested two different inoculation conditions (*i.e.* "Inoc (+)" and "Inoc (−)" for cell culture trials. The PC22A, PC168, PC170, PC173, PC182, PC21A (PE21 and PE103), and Iowa106 (PV39) strains grew in Vero cells only under the "Inoc (+)" condition, whereas PC177 grew only under "Inoc (−)" condition. PC179 and PC180 grew under both "Inoc (+)" and "Inoc (−)" conditions (Table 1). The time of CPE onset differed significantly among samples during initial isolation (1–5 days); however,
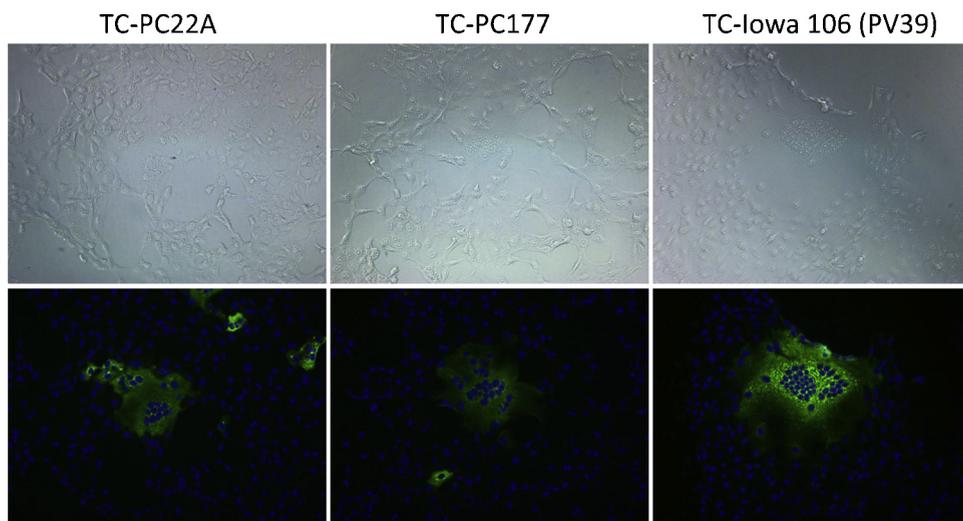

Fig. 1. PEDV strains, PC22A (highly virulent), PC177 (197 aa S deletion) and Iowa106 (sample #PV39, S INDEL) replicated in Vero cells. Upper and lower panels show light and the corresponding immunofluorescent microscope images, respectively, of Vero cells infected with the PEDV strains. PEDV-infected Vero cells were fixed at 2 PID. PEDV and nuclei were detected with mouse anti-PEDV N protein monoclonal antibody #6-29 and 4′,6-diamidino-2-phenylindole (DAPI), respectively. The infected cells showed obvious syncytial cells containing different numbers of nuclei (green for PEDV antigens, and blue for nuclei).

in the later passages, CPE was visible at 1–2 PID. Syncytial cells were not observed in mock-inoculated Vero cells.

We have confirmed that these nine isolated PEDV strains [PC22A, PC168, PC170, PC173, PC177, PC180, PC182, PC21A (PE103), and Iowa106 (PV39)] were all negative for other porcine enteric viruses [calicivirus, rotavirus groups A, B and C, deltacoronavirus, transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV)] by RT-PCR or real-time RT-PCR.

PC179 and PC21A (sample ID: PE21) strains were not used for subsequent passages in Vero cells because PC179 and PC180 were SIC and LIC samples from the same pig, respectively, and PE21 and PE103 were passage levels 1 and 4 of the PC21A strain in gnotobiotic piglets, respectively (Table 1) (Jung et al., 2014).

The viral RNA titers of the 9 PEDV strains [PC22A, PC168, PC170, PC173, PC177, PC180, PC182, PC21A (PE103), and Iowa106 (PV39)] propagated in Vero cells were similar (12–13 $\log_{10}$ GE/mL) (Table 1). The viral infectious titers ranged from 3 to 6 $\log_{10}$ PFU/mL.

The presence of PEDV antigens in infected Vero cells was confirmed using anti-PEDV N protein monoclonal antibody #6-29 (Fig. 1). The PEDV N protein was detected in the cytoplasm of typical syncytial cells (Fig. 1). Also typical coronavirus particles were observed by transmission electron microscopy (TEM) in ultra-thin sections of a PEDV-infected Vero cell (Fig. 2A) and in the cell culture media (Fig. 2B).

4.2. Primer set PEDV-20320F and PEDV-21816R amplified all isolated PEDV strains

We generated specific amplicons for the 9 serially passaged PEDV samples [PC22A, PC168, PC170, PC173, PC177, PC180, PC182, PC21A (PE103) and Iowa106 (PV39)] using primer set PEDV-20320F and PEDV-21816R. PC177 strain showed a shorter PCR product (860 bp) compared to those of the other 8 PEDV strains (approximately 1500 bp) (Fig. 3).

4.3. The nine cell-culture isolated PEDV strains had three distinct S protein sequences including one novel strain with a large deletion

Phylogenetic analysis demonstrated that the 8 PEDV isolates [with the exception of Iowa106 (PV39) strain] were clustered with the original US PEDV strains, and Iowa
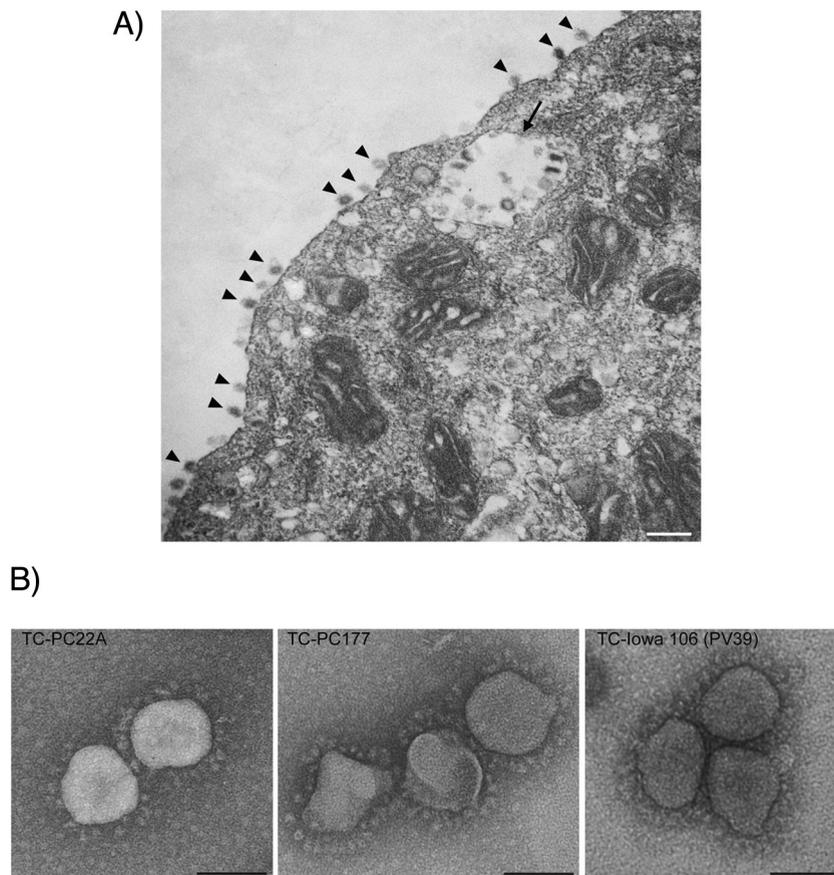

Fig. 2. (A) Electron micrograph of a PEDV-infected Vero cell. PEDV particles (arrow heads) on the cell surface and inside a vesicle (arrow) of infected Vero cell. Scale bar = 200 nm. (B) Electron microscopy images of PEDV virions from cell culture media of Vero cells infected with different PEDV strains: PC22A (highly virulent), PC177 (197 aa S deletion) and Iowa106 (sample #PV39, S INDEL). Scale bars = 100 nm.
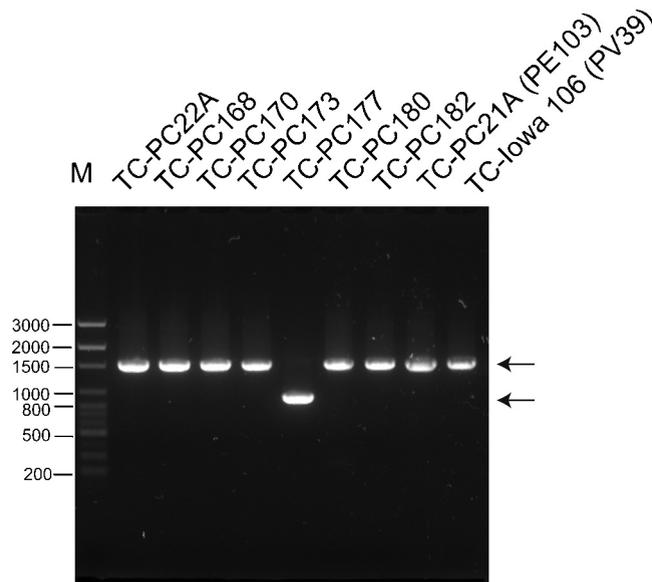
Fig. 3. PCR product patterns amplified by the versatile primer set (PEDV-20320-F and PEDV-21816R) targeting PEDV S protein gene. The RT-PCR products for virulent PEDV [PC22A, PC168, PC170, PC173, PC180, PC182, PC21A (PE103)], PC177 (197 aa S deletion) and Iowa106 (sample #PV39, S INDEL) are shown with arrows. M: DNA molecular size marker. The representative DNA sizes are shown in the left. The RT-PCR product size of PC 177 (approximately 860 bp) was obviously smaller than the others (approximately 1500 bp) as indicated by arrows.

106 (PV39) was clustered together with the S INDEL strain OH851 (Figs. 4 and 5). Further analysis indicated that the major differences among strains were mainly in the N-terminal region of the S protein (aa 1-388 according to USA/Colorado/2013 strain, GenBank accession no. KF272920) (Fig. 6). Based on the analysis of this region, the nine strains were distributed between two major distinct clusters: (1) a major cluster [PC22A, PC168, PC170, PC173, PC177, PC180, PC182, and PC21A (PE103)], similar to the original highly virulent US PEDV strains, *i.e.*, Colorado/2013; and (2) Iowa106 strain (PV39), similar to S INDEL US PEDV strain, OH851 (Genbank accession no. KJ399978) (Wang et al., 2014). Based on the partial S protein amino acid sequence alignment, PEDV strains PC22A, PC168, and PC170, which were collected from different farms, were identical, and those of PC173, PC180, PC182, and PC21A (PE103) had only one or two amino acid residue differences (data not shown). The PC177 had a large (197 aa) deletion in the N-terminal portion of the S protein compared to PC22A strain. Additionally, S INDEL Iowa106 strain (PV39) had minor insertions (2 aa) and deletions (5 aa) in comparison to the virulent PC22A strain, consistent with its designation as an S INDEL strain as previously reported (Fig. 6) (Vlasova et al., 2014; Wang et al., 2014).

5. Discussion

We initiated studies on cell culture isolation and characterization of the US PEDV strains in June 2013 and have successfully isolated nine PEDV strains out of 88 PEDV-positive samples. Recently, one paper reported the isolation of two US original PEDV strains (ISU13-19338E-IN and ISU13-22038-IA) using Vero-81 cells (Chen et al., 2014), shown in our phylogenetic trees (Figs. 4 and 5).

We have also isolated several PEDV strains [PC22A, PC168, PC170, PC173, PC180, PC182, and PC21A (PE103)] that genetically similar to ISU13-19338E-IN and ISU13-22038-IA strains (Fig. 4). For the first time, we successfully propagated S INDEL strain Iowa106 (PV39) similar to the reported US S INDEL PEDV strain (OH851) (Wang et al., 2014). In addition, we have isolated a third type of US PEDV strain, the PC177 that contains a large deletion (197 aa) in the predicted N-terminal region of the S protein compared to the PC22A strain (Figs. 5 and 6). Despite the 197 aa-deletion in the S protein, the PC177 strain grew in Vero cells to similar titers to those of other PEDV strains (Table 1), suggesting that this region is dispensable for virus infection/replication in Vero cells. The PC177 strain showed similar CPE but produced diffuse and faint (without a clear edge) plaques compared to other PEDV strains (data not shown). It is important to investigate the biological significance of this large deletion and the pathogenicity of this strain in pigs in future studies.

The nine isolated PEDV strains originated from intestinal contents of naturally infected pigs (field cases) or experimentally inoculated gnotobiotic or conventional pigs. Intestinal contents may be a better source for PEDV isolation because no PEDV was isolated from feces and serum in our study, although other factors may affect virus isolation in Vero cell cultures. Cell toxicity was observed for 6 intestinal content samples collected from field cases, and for two samples from the experimentally infected gnotobiotic and one conventional pig intestinal contents. Among the nine successfully isolated PEDV strains, eight PEDV strains could be isolated under "Inoc (+)" condition. Among the eight strains, only one strain (PC180 and PC179 were from the same pig) grew under both "Inoc (+)" and "Inoc (−)" conditions. No strain was isolated under "Inoc (−)" condition only except for PC177 which contains a
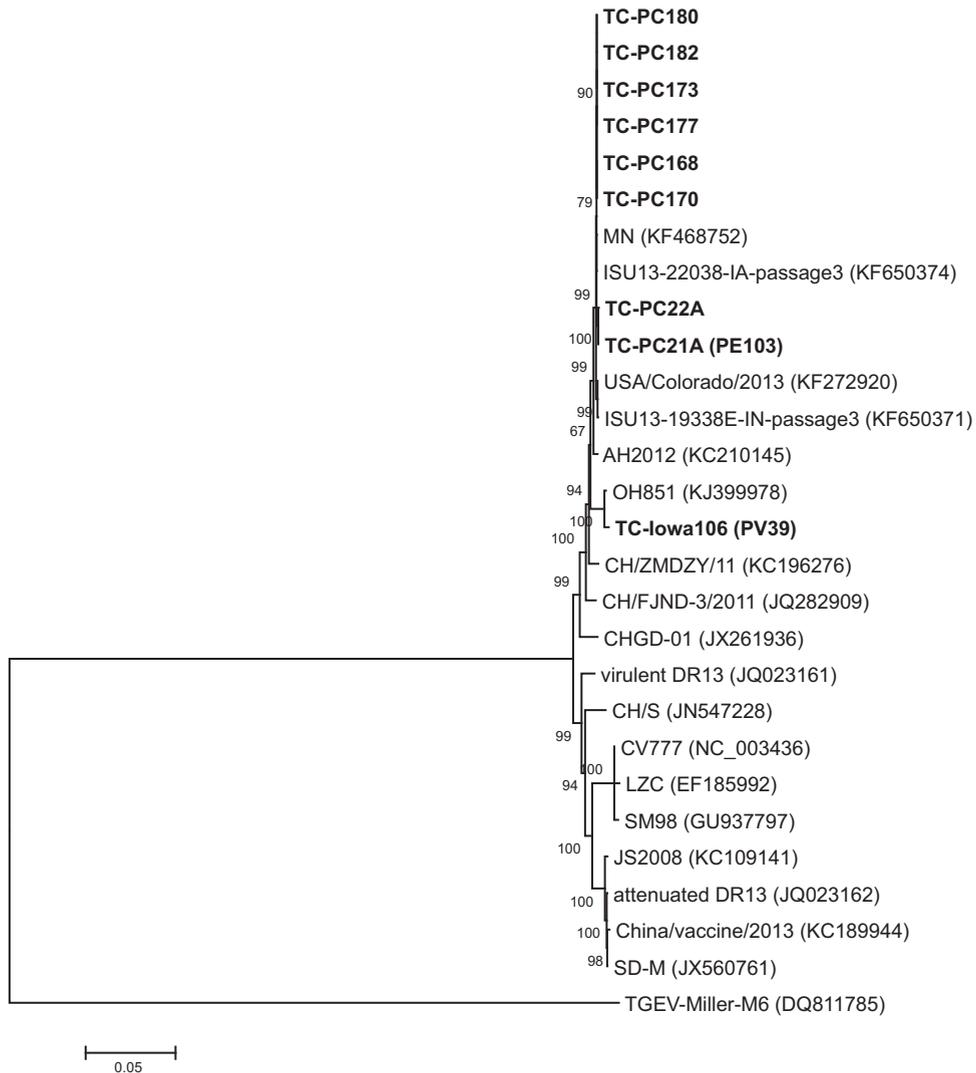
Fig. 4. The PEDV phylogenetic tree based on the nearly full genomic sequences were constructed using the Maximum Likelihood method with General Time Reversible nucleotide substitution model and supported with a bootstrap test of 1000 replicates in MEGA 6.06 software. The numbers on each branch indicate the bootstrap values. The scale represents the nucleotide substitutions per site. The 9 PEDV strains isolated in this study were bolded.

197 aa-deletion in the S protein. These results indicate that multiple factors, such as the type of sample, virus titer, substances in intestinal contents, and strain-specific factors may affect virus isolation. Using different Vero cell lines and culture conditions may improve PEDV isolation rates from clinical samples.

We isolated different PEDV strains at different times during one year. The PC22A strain was isolated first and we continued to passage it in Vero cells to high passages. The PC21A strain was isolated later using pig-passaged sample (PE103). The PC21A and PC22A strains were originally from two individual pigs that died from the same PEDV outbreak in 2013 and they were genetically similar. Therefore, we did not further passage the PC21A strain. Recently, we isolated PC168-PC182 strains and the S INDEL strain Iowa106 in Vero cells. Among them, we selected PC177 and Iowa106 strains for further serial passaging because those two strains had distinct S protein sequences.

All the isolated PEDV strains had similar RNA titers ($12–13 \log_{10}$ GE/mL) at different passage levels (Table 1). However, the viral infectious titers ranged from 3 to 6 $\log_{10}$ PFU/mL. We noticed that during continuously passaging of PC22A, PC177 and Iowa106 (PV39) strains, although infectious titers by plaque assay increased after several more passages (data not shown), their viral RNA titers stayed almost unchanged after passage 2. The reason could be that some viral RNA detected was from defective particles or other viral particles that cannot form plaques at the beginning of virus isolation. After several passages, the virus strains adapted to cell culture better and the majority of virus population form plaques.

The primers we designed in this study target the conserved region spanning the non-translated region between ORF1b and the S gene (approx. 295 nt) and the N-terminal part of the S gene (approx.1165 nt). This primer set detected all 9 PEDV isolates described in this study
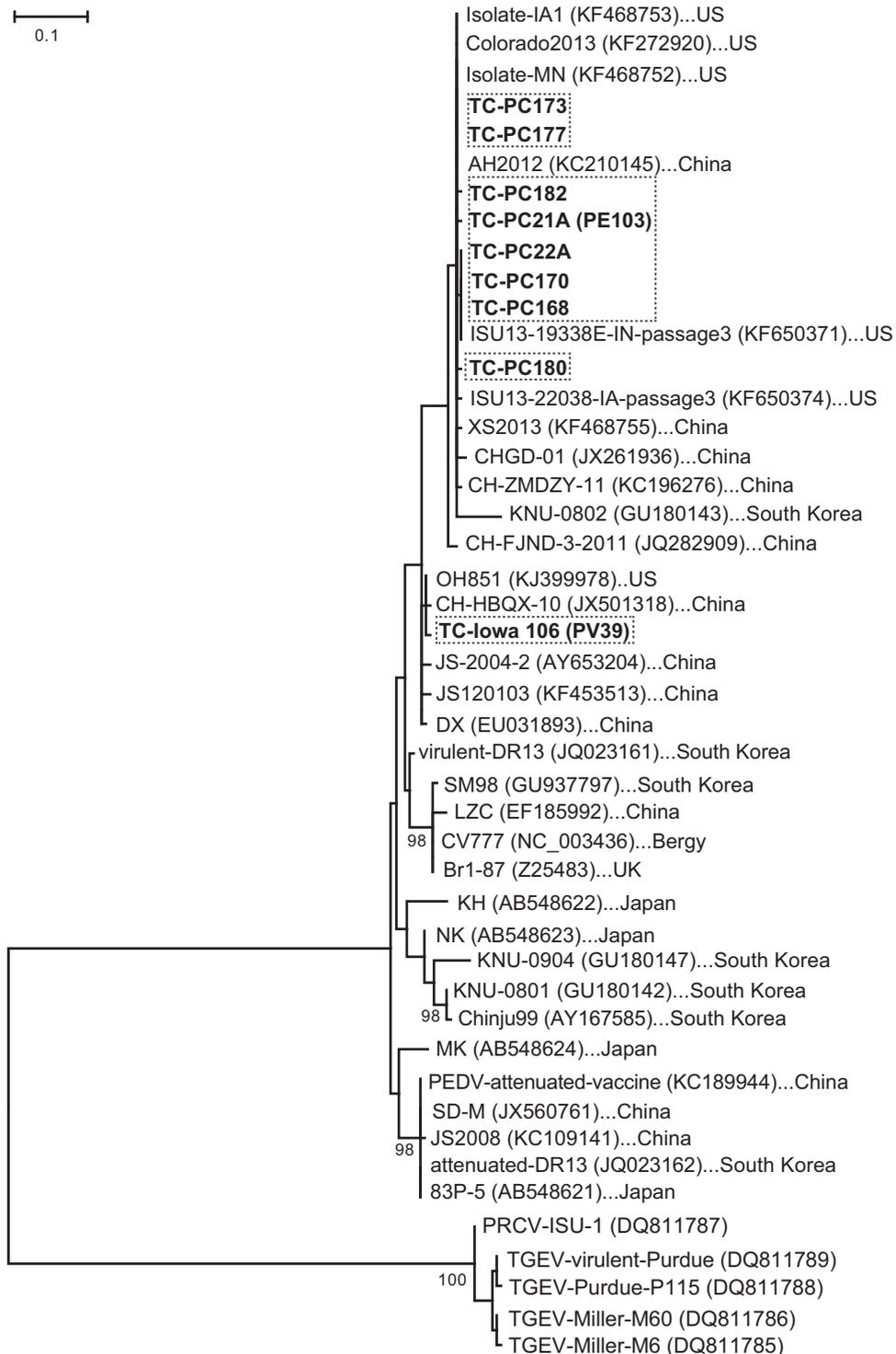
Fig. 5. The phylogenetic tree based on partial S proteins (aa 1-388 according to USA/Colorado/2013 strain, GenBank accession No. KF272920) was constructed using the Maximum Likelihood method based on the JTT model. Nine PEDV strains isolated in this study and 31 reference PEDV strains were included, TGEV and PRCV were used as outgroup controls. Genbank accession numbers for reference PEDV, TGEV, and PRCV strains are shown in parenthesis after each strain name. Bootstrap was performed with 1000 replications. The numbers on each branch indicate the bootstrap values of ≥95%. The scale represents the amino acid substitutions per site. The 9 PEDV strains isolated in this study were subdivided into two groups based on partial S protein characteristics: (1) virulent PEDV [PC22A, PC168, PC170, PC173, PC182, PC180, PC21A (PE103)], PC177 (197 aa S deletion); and (2) Iowa106 (sample #PV39, S INDEL) within dashed boxes.
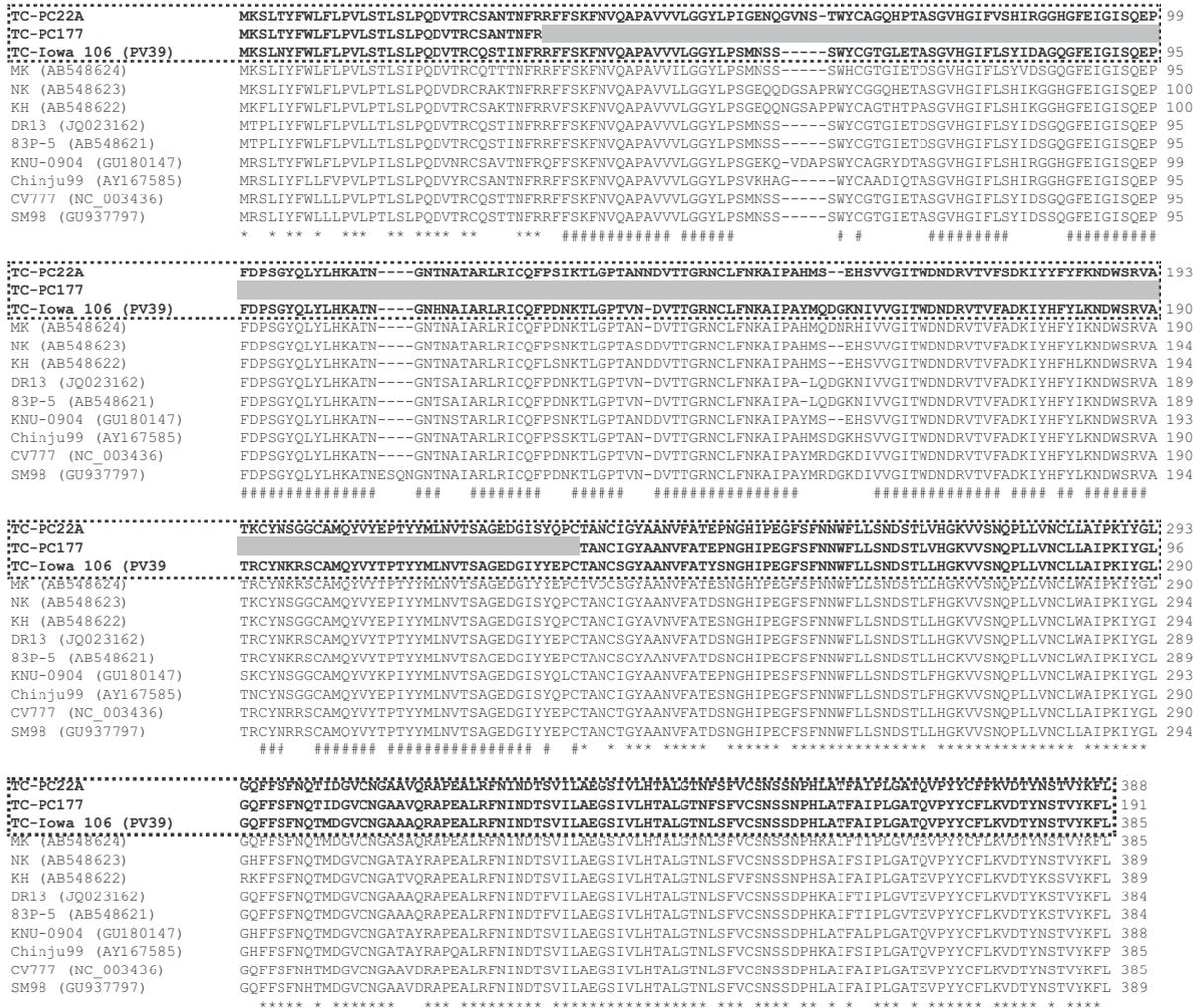
Fig. 6. Amino acid sequence alignments of partial S protein (1–390 aa) of 12 representative PEDV strains. Genbank accession numbers are shown in parenthesis after each strain name. The representative 3 PEDV strains [PC22A (virulent PEDV), PC177 (197 aa S deletion), and Iowa 106 (sample #PV39, S INDEL)] isolated in this study are indicated within dashed boxes and bold letters. Compared to PC22A strain, PC177 has a large 197aa deletion (highlighted in gray bar) and Iowa106 (PV39) has a minor insertion and deletion compared to the PC22A strain. Deletion is labeled as "−". The conserved amino acid residues among the 11 or 10 (except for PC177) representative strains are labeled as "*" or "#".

(Fig. 3), which are genetically diverse: (1) PC22A, PC168, PC170, PC173, PC180, PC182, and PC21A (PE103) strains; (2) Iowa106 (PV39) strain; and 3) PC177 strain with the unique large deletion in the N terminal region of the S protein) (Figs. 5 and 6). To our knowledge, this is the first report of a strain with a large deletion (197-aa) in the S protein compared to the US major cluster strains. In contrast, the nucleotide sequences between the primer PEDV-20320-F and the nucleotide before the start codon of the S protein gene were highly conserved [only 1 nt difference in Iowa106 (PV39)] among the 9 PEDV strains, and are not suitable to distinguish the genetic differences.

The unique characteristic of the S gene of PC177 strain was first identified by the ∼600 bp-shorter PCR product size (Fig. 3). In contrast, the other 8 PEDV isolates generated similar PCR product sizes. Nucleotide sequence analysis is necessary to distinguish strains from the two

previously reported PEDV clusters (*i.e.*, the clusters similar to the major US highly virulent strains and the reportedly S INDEL strains) (Figs. 4 and 5), because there are only 9-nt (corresponding to 3 aa) differences in length between these two clusters of strains in the RT-PCR target region (Fig. 6). We showed that the primer set, PEDV-20320F and PEDV-21816R amplifies a target fragment from the genetically diverse PEDV strains such as the ones isolated in this study, which have highly variable sequences in the N-terminal region of the S protein.

Sequence alignment showed that the N-terminus of the S protein of all the US PEDV strains (data not shown), including the nine strains from this study, was initiated from "MKS". The PC22A, PC177, and Iowa106 (PV39) partial sequences are shown as representative strains in Fig. 6, while the vaccine European strain CV777 or Asian strains (DR13 and 83-P5) start with "MRS" or "MTP",

respectively (Fig. 6). These results suggest that the current PEDV strains detected in the US differ from the European and Asian vaccine strains in this region. The PEDV strains starting with "MKS" were also reported from China [AH2012, XS2013, CHGS, CH-ZMDZY-11 and CH-FJND strains clustered together with US highly virulent PEDV strain (Colorado/2013), and CH-HBQX-10, JS2004-2, JS120103 and DX clustered together with US reportedly mild PEDV S INDEL strain OH851] (Fig. 5), suggesting global circulation of these two types of strains at least in the US and China. Interestingly, the KNU-0802 strain found in South Korea clustered together with the US highly virulent strains with "MRS" in the beginning of the spike and differed from the strains reported from the US and China.

We did not detect the large S deletion in the original PC177 sample, but the large deletion was detected in the first (P0) cell culture passage under "Inoc (−)" conditions (Materials and Methods). There are at least two possible explanations: (1) a minor proportion of PEDV strains that contained this large S deletion was initially present in the original PC177 sample and was more replication competent in Vero cells; or (2) the large S deletion occurred during the initial Vero cell culture *via* an unknown mechanism. The former possibility is consistent with the recognition that coronaviruses exist naturally as quasi-species or mixed populations of strains (Zhang et al., 2007). However, we did not detect the large S deletion in the original PC177 sample by using deep genome sequencing (data not shown), indicating the large S deletion occurred during cell adaptation. It is important to further investigate if the large S deletion strains circulate naturally in US swine by using the primer sets we designed in this study.

The pathogenicity of the major highly virulent US PEDV cluster strains has been reported recently (Jung et al., 2014; Stevenson et al., 2013). Pathogenesis studies of different PEDV isolates may aid in identification of mild/attenuated PEDV strains, which can be used for the development of attenuated PEDV vaccines. Interestingly, the deletion location and size identified in the PC177 strain is similar to that in other porcine coronaviruses: the TGEV-mutant PRCV-ISU-1 strain has a 227 aa-deletion in the N-terminal portion of the S protein (Zhang et al., 2007) and as a result has a different tissue tropism (respiratory infection) and produces an attenuated disease compared to the parental TGEV strain (Saif et al., 2012). Although PC177 was isolated from a diarrheic fecal sample, it is important to test whether the PC177 strain also possesses respiratory or dual (pneumo-enteric) tropism and shows reduced virulence.

Multiple US PEDV strains adapted to cell culture will provide critical new reagents to develop US PEDV vaccines, to study the biology of PEDV strains, and to develop PEDV immunoassays, virus inactivation efficiency assays and screen for PEDV antivirals. The S gene primer set designed in this study can be used for identification of different PEDV strains from field samples. The highly virulent PEDV has now spread throughout North America and also in Asia, despite the availability of inactivated and attenuated PEDV vaccines, CV777 in China and DR13 in Korean, respectively. Therefore, it is important to investigate PEDV variants currently circulating in these countries, to assess their ability to cross-protect against highly virulent PEDV strains and to find ways to prevent and control PEDV epidemics.

In conclusion, we successfully isolated PEDV strains from each genetic cluster detected in the US (*i.e.*, major highly virulent cluster, and reportedly mild S INDEL cluster). Additionally, we also isolated a PEDV strain (PC177) that has a large deletion in the S gene. These culturable PEDV strains are critical for future pathogenic, antigenic, and molecular biological studies. Also, they can be used to develop the much needed PEDV vaccines.

Acknowledgments

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